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Evaluating an interspecific *Helianthus annuus* × *Helianthus tuberosus* population for use in a perennial sunflower breeding program

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ABSTRACT

Perennial crops show promise for sustainable agricultural production while providing ecosystem services (maintaining healthy soil, controlling erosion, improving water quality, and enhancing wildlife habitat). Perennial crops could also provide economically viable cropping option to farmers. Sunflower (*Helianthus annuus* L.) is an ideal crop for perennialization because of existing genetic resources and a wide variety of end-uses. The objective of this research was to evaluate interspecific hybrids between perennial *Helianthus tuberosus* L. ($2n = 6x = 102$) and annual *H. annuus* L. ($2n = 2x = 34$) for perenniality and agronomic traits; assessing their utility in developing a perennial seed crop. Field trials indicated that seed yield traits were positively correlated with head traits. Tuber traits, which are required for perenniality, and seed yield traits were not correlated, indicating that simultaneous selection may be able to target high yielding lines that also tuberize. The F_1 individuals were intermated for one generation and the intermated F_1 (IM_1F_1) showed increases in head size (up to 20%) compared to the best F_1 individual. The lack of correlation between tuber and seed traits coupled with phenotypic improvement after one generation of intermating suggest that the best improvement strategy for perennial sunflower is a recurrent selection program focusing on yield.

1. Introduction

Over the past century, agricultural research has contributed to dramatically increased crop yields and productivity, yet this increase in productivity has often come at the expense of long term environmental sustainability through greater use of fossil fuel-based fertilizers, the depletion of fresh water, and the reduction of arable land (Baulcombe et al., 2009; Tilman et al., 2002). Addressing environmental damage and enhancing ecosystem services such as climate regulation, water management, and soil fertility will be essential for the adequate production of food in the future (Baulcombe et al., 2009; Costanza et al., 1997; Tilman et al., 2002). Currently there are cultural practices such as zero tillage and cover cropping, which provide many ecosystem services without a yield reduction. Recently, the addition of perennial plants, particularly perennial crops, has been suggested as another tool for incorporating ecosystem services into the landscape while maintaining productivity (DeHaan et al., 2005; Baulcombe et al., 2009; Glover et al., 2010; Chia et al., 2012). The potential of perennial crops to reduce the environmental impact of agricultural systems through reduction in fall tillage, soil erosion, and nutrient runoff has long been ignored, but recently has regained popular interest (Glover et al., 2010). In addition, due to reduced input costs, perennial grains can be as profitable as annual counterparts over a three year life of the perennial crop if the market price is equal and the perennial yields at least 60% as much as the annual crop (Bell et al., 2008).

Recent research has shown that the genetics of perenniality (development of perennial organs) may not be as complex as previously thought, with several studies identifying only a few quantitative trait loci (QTL) necessary for perennial organ development (Wang et al., 2009; Sacks et al., 2007; Hu et al., 2003). Hu et al. (2003) identified two QTL that controlled production of rhizomes in rice (*Oryza longisteminata* × *Oryza sativa* hybrids) with the segregation matching a dominant two gene model with complementary gene action. Stolon presence:absence segregated in a 3:1 fashion in F_2 families of the interspecific cross *O. sativa* × *O. rufipogon* (Sacks et al., 2007). Further, Wang et al. (2009) identified a single gene, perpetual flowering 1 (*PEP1*), which regulates

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perennial flowering in *Arabis alpina*. These findings suggest it may be possible to introduce perennial habit into annual crops without introducing large portions of wild relatives of the species.

Domesticated sunflower (*Helianthus annuus* L., $2n = 2x = 34$) is an annual crop that produces a diverse range of products, including oilseed types (used to produce birdseed or high-quality vegetable oil) and confection seeds for direct human consumption. Sunflower is a compelling target for perennialization, as *Helianthus* includes 49 species, many of which are perennial (Kane et al., 2013). Breeders have used interspecific hybridization to introgress useful wild traits into *H. annuus* for disease resistance (Miller and Gulya, 1987), insect resistance (Charlet and Brewer, 1995), adaptation to distinct environments, abiotic stress (Rieseberg, 1997), and cytoplasmic male sterility (Kohler and Friedt, 1999). A similar approach could be used to introgress the perennial habit, as perennial *Helianthus* species can potentially be used as donor materials for transferring perennial habit into domesticated sunflower. Breeding for perennial seed crops poses a unique problem because perennials need to allocate photosynthetic resources to both the perennial organs used for carbon storage and the seed itself (DeHaan et al., 2005). It has been suggested that perennial plants can be selected for increased seed production while maintaining asexual reproduction (Cox et al., 2002; DeHaan et al., 2005). Perennial plants have a longer growing season to assimilate nutrients and breeding can influence photosynthate utilization to optimize seed production and perennial habit. In addition, there is historical precedent as farmers who initially domesticated rice selected for perennial habit during low intensity production (Hill, 2010).

Helianthus tuberosus, a tuber-bearing perennial species, is a prime candidate for the introduction of perenniality into domestic sunflower. It has been used to introgress traits into *H. annuus* for nearly a century and has a separate history as a specialty crop (Hulke and Wyse, 2008). *H. tuberosus* ($2n = 6x = 102$), is an autoallohexaploid with three sub-genomes. The three sub-genomes have been traditionally designated as A_1, A_2, and B_t (Kostoff, 1939). The B_t sub-genome is thought to be very similar to the *H. annuus* genome (Kostoff, 1934, 1939; Scibria, 1938), which may help stabilize meiotic chromosome pairing in interspecific hybrids between the two species. Through conventional hybridization it is possible to create large populations of interspecific *H. annuus* × *H. tuberosus* hybrids. The hybrids are perennial by way of tuber-sprouting and have good fitness. Commercial varieties have been released in Russia and Sweden for tuber production and forage purposes (Kays and Nottingham, 2008). *H. annuus* × *H. tuberosus* hybrids generally have a stable intermediate number of chromosomes ($2n = 4x = 68$), although meiotic abnormalities can reduce fertility and decrease stability in initial generations (Sujatha and Prabakaran, 2006; Chandler et al., 1986; Atlagic et al., 1993).

Three breeding strategies have been proposed to create perennial grain crops: direct domestication of perennial relatives of crop plants, transgenic modification of annual plants, and genetic introgression of perennial habit from wild relatives into domesticated crops through wide hybridization (Glover et al., 2010). There is doubt regarding the feasibility of direct domestication of perennial sunflower relatives as QTL mapping studies within annual sunflower suggest that a larger number of loci contribute to domestication-related phenotypes in sunflower than in other species (Burke et al., 2002; Wills and Burke, 2007; Doebley and Stec, 1991), Furthermore, the most important domestication trait in sunflower is suppression of axillary flowers (single headed state) (Chapman et al., 2008), a trait present at low frequencies in wild sunflower populations. Transgenic modification is not possible at present, as no known "perenniality" genes have been identified for sunflower, and only one has been identified so far in other species (Wang et al., 2009). Moreover, sunflower is recalcitrant to regeneration and transformation (Lewi et al., 2006; Piqueras et al., 2010), and gene flow issues with weedy conspecifics have halted regulatory acceptance of transgenic sunflower (Snow et al., 2003). Introgression of perennial habit from wild relatives through wide hybridization may be the most feasible approach. The main advantage of this approach is that a copy of the domesticated genome is present in a hybrid, enabling the selection of existing domesticated or elite loci that may not be present at high frequency in the wild germplasm. This approach can be implemented in at least two different ways: (1) selection on a population backcrossed to the domesticated parent or; (2) recurrent selection on populations derived from intermating the hybrid materials.

This study evaluates an interspecific population of *H. tuberosus* × *H. annuus* as a base for developing a perennial oil-seed sunflower. We validate the interspecific origin of hybrid populations, examine parental diversity, and then evaluate the potential for improving the perennial populations based on the interactions between perennial, agronomic, fertility and yield traits.

2. Materials and methods

2.1. Populations

Five populations were investigated. The first population was 18 *H. tuberosus* individuals collected from UMore Park in Rosemount, MN. The second was a set of 187 interspecific F_1 hybrids between *H. annuus* and *H. tuberosus*. The interspecific hybrids were developed during the years 2003–2006 (Hulke and Wyse, 2008) by crossing the 18 *H. tuberosus* (perennial) parents with three inbred *H. annuus* (annual) lines (CMS HA 89 [PET1], HA 89 (released by the USDA-ARS in 1971) and HA 434 (Miller et al., 2004)). HA 89 and HA 434 were used as male parents and CMS HA 89 was used as a female parent (Supplementary Table 1). The third population was a derivative of the second, as the F_1 hybrids were intermated to form an intermated F_1 population (designated as the IM_1F_1 population). This population was developed in 2007 by Hulke and Wyse (2008) (Supplementary Table 1). The fourth population was a backcross of the interspecific F_1 to the inbred lines HA 434 and HA 89 (designated as the BC_1F_1 population). This population was developed in 2006 by Hulke and Wyse (2008) (Supplementary Table 1). The fifth population was 31 *H. tuberosus* plants from the seed stocks of the United States Department of Agriculture Germplasm Resources Information Network (GRIN) that were collected from a diverse set of geographical locations (Supplementary Table 1) (USDA, 2012).

2.2. Flow cytometry

Individuals in the following populations were examined for genome size using flow cytometry: 187 interspecific F_1s, 170 IM_1F_1s, 120 BC_1F_1s, the 18 *H. tuberosus* parental lines and two of the *H. annuus* parental lines (HA 89 and HA 434).

Nuclear DNA content was assessed using a BD FACSCalibur (BD Biosciences, San Jose, CA) flow cytometer. Two technical replicates of the same clone were performed (on different days) for each plant on 42 F_1 individuals and the inbred annual lines. A single measurement was performed on the other individuals. Fully expanded leaf tissue sections of 0.55 cm^2 were finely chopped in 500 ml of extraction buffer (Partec, CyStain PI Absolut P), followed by filtration through a 50 micron nylon mesh. Filtered nuclei were stained with 2 ml of propidium iodide staining solution (Partec, CyStain PI Absolut P), stored at 4 °C, and examined within 12 h of preparation. A commercial standard of trout erythrocytes (Partec, DNA Control UV, 25 ml) as well as the internal standard from diploid HA 89 were used to calculate DNA content. A minimum of 1000 nuclei were examined for each sample. DNA content was calculated by taking the ratio of the peak intensity of each sample to that of the known standard and then multiplying the ratio by the picogram (pg) genome

Table 1
This table describes the phenotyping methodology for each trait examined.

Days to flowering	Counted from date of emergence in the spring to the appearance of the first flower
Total tuber number Total tuber weight (grams) Average tuber weight (grams)	Tubers were harvested in November 2009 by digging, washing, counting and weighing all tubers in a 0.5 m radius around each individual plant, as we observed that most tubers were centered in this area. To improve efficiency in data collection, in 2010, a subsample was taken at Rosemount and St. Paul that consisted of six 15 cm soil cores taken in a 0.5 m radius around each individual plant. The subsamples were calibrated to whole plot measurements by harvesting a row of plants (41 in total) in 2010 using the 2009 method. When the measurements of all tubers per plant in 2009 were compared to the partial sampling measured in 2010, the $R^2 = 0.71$ for tuber number and $R^2 = 0.63$ for tuber weight (based on individual plant), with the relationship between years fitting a linear model better than a quadratic model
Pollen viability	Scored by staining pollen with Alexander stain and scoring 300 pollen grains from each replication (Alexander, 1980)
Head number	Counted for each plant at physiological maturity
Branching type	Scored on a scale of 0-4 according to Hockett and Knowles (1970), with 0 being no branching
Spreading ability	Above ground plant spreading ability was scored on a 1-5 scale with 1 indicating the domestic phenotype of spreading to 15 cm, 2 indicated that plants spread 15-30 cm, 3 indicated intermediate spreading of 31-60 cm, 4 indicated vigorous spreading of 61-90 cm, 5 had spread greater than 90 cm
Maximum head diameter (cm)	Measured in cm after plant physiological maturity
Average head diameter (cm)	Measured in cm after plant physiological maturity. Ten randomly selected heads, including the central head, were measured to calculate average head diameter
Number of seeds per head	Calculated by dividing the total number of seeds by the ten heads harvested
Seed weight (grams)	Calculated by threshing ten random heads from each plant, including the central head, and weighing the resulting seeds. Heads were randomly chosen on plants. All plants were not harvested at the same time but all heads were harvested from the same individual plant on the same day. Plants were harvested as they reached physiological maturity, so early maturing individuals were harvested earlier
Average seed weight (grams)	Calculated by weighing the seed from the ten heads and dividing by the total number of seed

size of the standard. The BC_1F_1 populations were characterized with freeze dried tissue, which has decreased fluorescence relative to fresh tissue (Doležel et al., 2007). Freeze dried samples were calibrated by identifying differences between freeze dried and fresh tissue of the same clone in a subsample of 40 F_1 individuals. Ploidy boundaries were assessed by constructing 95% confidence intervals around the mean genome content of each population and comparing the genome content to the known values in the literature. Confidence intervals were constructed by 1000 bootstrap replications from the empirical distribution of each population. Briefly, a random sample was drawn with replacement from genome sizes in each population, creating a new distribution from which the 2.5% and the 97.5% individuals were used to create a 95% confidence interval for each ploidy level (Efron and Tibshirani, 1993).

2.3. Phenotyping of the interspecific populations

2.3.1. F_1 phenotyping

The 187 F_1 hybrids (*H. tuberosus* × *H. annuus*) and 18 *H. tuberosus* parents were field grown in St. Paul (2009 and 2010) and Rosemount, Minnesota (2010). Plants were grown in a randomized complete block design with three replications per environment. The two environments were separated by approximately 35 km and differed in climate and soil: St. Paul (located at 45°00′ N 93°05′ W), has a soil type of fine-silty over sandy or sandy-skeletal, mixed, mesic Typic Hapludolls, and Rosemount (located at 44°44′ N 93°01′ W), has a soil type of a well-drained Waukegan silt loam (fine silty over sandy, mixed mesic Typic Hapludolls). F_1 hybrids were transplanted as young plants newly emerged from tubers in May in both St. Paul 2009 and Rosemount 2010 from a living collection maintained in St. Paul. F_1 hybrid tubers were harvested from the St. Paul 2009 planting and replanted in a different field in St. Paul in November 2009. All plants were grown one meter apart within rows and 1.8 meters apart between rows. Large tubers were used for planting, as sprouting and survival increase with larger tubers (Kays and Nottingham, 2008). Plants were scored for 13 traits. The traits and phenotyping procedures are outlined in Table 1.

Statistical analysis was conducted using the R Statistical software package (R Development Core Team, 2012). Analysis of variance was conducted using families and individuals as fixed effects and environment as a random effect. Replications were nested within environment. A significance level of $\alpha = 0.05$ was used to determine significant differences. Contrasts were performed with PROC GLM of SAS to test differences between *H. tuberosus* parents and F_1 progeny (SAS Institute, 2008). Means, ranges, and heritability were calculated for each trait evaluated and phenotypic correlations estimated for each pair of traits. Narrow sense heritability was calculated using the 18 *H. tuberosus* half-sib families within each environment and then pooled across environments using parent offspring regression (Fehr, 1991). Parent-offspring regression coefficients were calculated by 1000 bootstrap replications from the residuals of an initial regression where wild parental values were used to predict the value of the interspecific progeny (Efron and Tibshirani, 1993).

2.3.2. IM_1F_1 phenotyping

F_1 individuals were intermated to generate an IM_1F_1 population. In 2009, 71 IM_1F_1 plants were grown in St. Paul. The 55 surviving IM_1F_1 plants were phenotyped for average head diameter, largest head diameter, pollen viability, seeds per head, seed yield and individual seed weight in 2010 in St. Paul. During the winter of 2010–2011, 151 IM_1F_1 plants were grown in the greenhouse in St. Paul and screened for tuber production, along with *H. tuberosus* plants as controls. Plants in the greenhouse were grown in 30 cm pots with 50–50 mix of Sunshine professional growing mix® (Sun Gro) and soil. Plants were grown in a greenhouse with 14 h day-length at 24 °C, they were given no supplemental light. In 2011–2012, 104 tuber-bearing IM_1F_1 plants out of the 151 IM_1F_1 plants were transplanted in late May and grown in the field at Rosemount. As the IM_1F_1 population exhibited segregation for perennial habit and tuber traits, Chi-square tests were used to test examine segregation ratios for tuber presence in the IM_1F_1 population. We tested a single gene model where the expected ratio is 3:1 and a two gene model having complimentary gene action where the expected ratio is 9:7, both cases assumed disomic inheritance and equal initial allele frequencies.

2.4. Parental diversity analysis

Thirty-one additional *H. tuberosus* accessions from the GRIN collection (Supplementary Table 1) were used to assess the diversity of the *H. tuberosus* germplasm, and estimate the representation of this diversity in our 18 lines used for breeding. DNA was prepared from the 31 accessions, along with 14 of the *H. tuberosus* and two of the *H. annuus* breeding parents, to assess diversity using molecular markers. Genomic DNA was isolated from fresh or freeze dried leaf

Table 2
1C DNA content ranges (pg) for *Helianthus annuus* and *H. tuberosis* accessions in the perennial sunflower breeding program and their F_1, intermated F_1 (IM_1F_1), and F_1 from first backcross to H. annuus (BC_1F_1) derivatives that were tested and literature values.

Accession	No. individuals	Range (pg)	Mean (pg)	Mean (Gb)	Reported Value (pg)[†]
HA89	108	3.14–3.82	3.45	3.37	1.78–3.98
HA434	98	3.15–4.11	3.6	3.52	1.78–3.98
Helianthus tuberosus	18	12.95–15.58[*]	14.52	14.20	12.55[‡]
F_1	187	6.92–16.92	9.98	9.76	NA
IM_1F_1	170	7.53–19.03	9.5	9.29	NA
BC_1F_1	120	4.89–6.28	5.45	5.33	NA

[†] Reported values are all based flow cytometry for *H. annuus* and on Feuglen Densitometry for *H. tuberosus*, and were reported in Bennett and Leitch (2010). Conversion to base pairs was done using the equation from Doležel et al. (2007).
[‡] Feugelin microdensitometry was used in the initial measurement, which may underestimate genome size in the presence of secondary metabolites (Doležel et al., 2007).
[*] Variation in genome size was greater among the hexaploid *H. tuberosus* individuals than among *H. annuus*, similar to high ploidy accessions of switchgrass (Costich et al., 2010).

tissue on all accessions using either a Qiagen Plant DNeasy Mini kit according to the manufacturer's protocol or a modified CTAB procedure optimized for sunflower (Webb and Knapp, 1990).

Sixteen expressed sequence tag-simple sequence repeat (EST-SSR) markers previously identified by Heesacker et al. (2008) were polymorphic in our population and used to genotype. Following DNA extraction, samples were sent to Biogenetic Services, Inc. (Brookings, SD). Samples were multiplexed by combining two loci, each with a different color label into a single plate. Multiplexed samples were loaded into the ABI3100 genetic analyzer and were run according to the manufacturer's standard recommendation. Direct labeled primers tagged with ABI dyes 6-FAM or Hex were scored on an Applied Biosystems Inc. (ABI) 3730xl capillary instrument. The resulting electropherograms were scored using the GeneScan software package (ABI). A numerical base-pair size was assigned to each electropherogram peak. The program TANDEM (Matschiner and Salzburger, 2009) was used to bin raw allele sizes. The program PowerMarker (Liu and Muse, 2005) was used to analyze genotype data by calculating expected heterozygosity, observed heterozygosity and the average number of alleles per locus. The SSR markers utilized appeared to follow a stepwise mutation model therefore we used R_{ST} to differentiate the populations. R_{ST} is a measure of population differentiation that accounts for SSR markers undergoing a stepwise mutation model (Slatkin, 1995). The stepwise mutation model postulates that an SSR marker will change (gain or lose) by only one repeat unit per generation (Di Rienzo et al., 1994). R_{ST} was calculated between *H. annuus* parents, *H. tuberosus* parents, and GRIN accessions utilizing the program GENEPOP (Rousset, 2008). The genetic distance between population pairs was calculated using the Nei73 coefficient (Nei, 1973). An unrooted neighbor-joining tree was constructed using MEGA 5 (Tamura et al., 2011). Genetic assignment of genotypes was performed with Structure version 2.3.4 (Pritchard et al., 2000). STRUCTURE was run with a Markov Chain Monte Carlo (MCMC) burn-in of 20,000 steps, followed by an MCMC chain of 10,000 steps for clustering inference. The number of subpopulations was determined by performing ten runs for each K (number of subpopulations), with $K=1$ to $K=8$ examined. An admixture model (mixed ancestry from multiple populations was allowed) was used along with uncorrelated allele frequencies between subpopulations. We used StructureHarvester to identify the optimum K using the Evanno method (Earl and vonHoldt, 2012; Evanno et al., 2005). CLUMPP was used to integrate results across runs per K (Jakobsson and Rosenberg, 2007).

3. Results

3.1. Validation of interspecific origin of hybrid populations

We utilized flow cytometry to compare the genome sizes of hexaploid *H. tuberosus* and diploid *H. annuus* to the hybrid offspring. A portion of the putative hybrid individuals may have resulted from inadvertent self-pollinations or mating between *H. tuberosus* individuals. Therefore, this analysis served to identify the individuals that were true interspecific hybrids (i.e., tetraploid plants).

The estimated 1C genome size of *H. annuus* is ~3.3 pg of DNA (Bennett and Leitch, 2010). The two diploid annual parents used in the present study exhibited similar values, as HA 89 and HA 434 were measured at 3.45 pg and 3.60 pg, respectively (Table 2). The average 1C genome size among the 18 *H. tuberosus* accessions was 14.52 pg, higher than the previously reported value of 12.55 pg (Bennett and Leitch, 2010).

Based on flow cytometry measurements, the expected genome size for an average tetraploid hybrid was approximately 9 pg. The vast majority of the putative F_1 hybrid plants (166 out of 187) exhibited flow cytometry readings near this value, indicating that these are likely true interspecific hybrids (Fig. 1). However, 21 putative F_1 hybrids (12.2%) had DNA content equal or greater than the reported 1 C genome size for *H. tuberosus* (12.55 pg). We inferred that these plants were hexaploid, resulting from either self-pollinated or intermated *H. tuberosus* plants. These plants were excluded from further analysis. In the intermated F_1 (IM_1F_1) population, nearly all plants (167 out of 170) contained approximately 9 pg DNA per cell, indicating that these plants maintained a tetraploid chromosome number. Three individuals (1.7%) in the IM_1F_1 population had a genome size outside the expected range (two appeared pentaploid, the other octoploid) and were also excluded from the phenotypic analysis. All BC_1F_1 plants were triploid and displayed annual habit. As no perennial plants were recovered, the BC_1F_1 population was not investigated further.

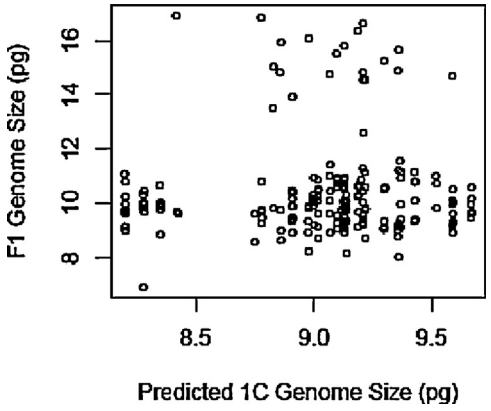

Fig. 1. The relationship between observed and predicted F_1 genome size based on the average of the two parents for each cross. Individuals that had a genome size above 12 pg were considered to be hexaploid and were discarded from phenotypic analysis.

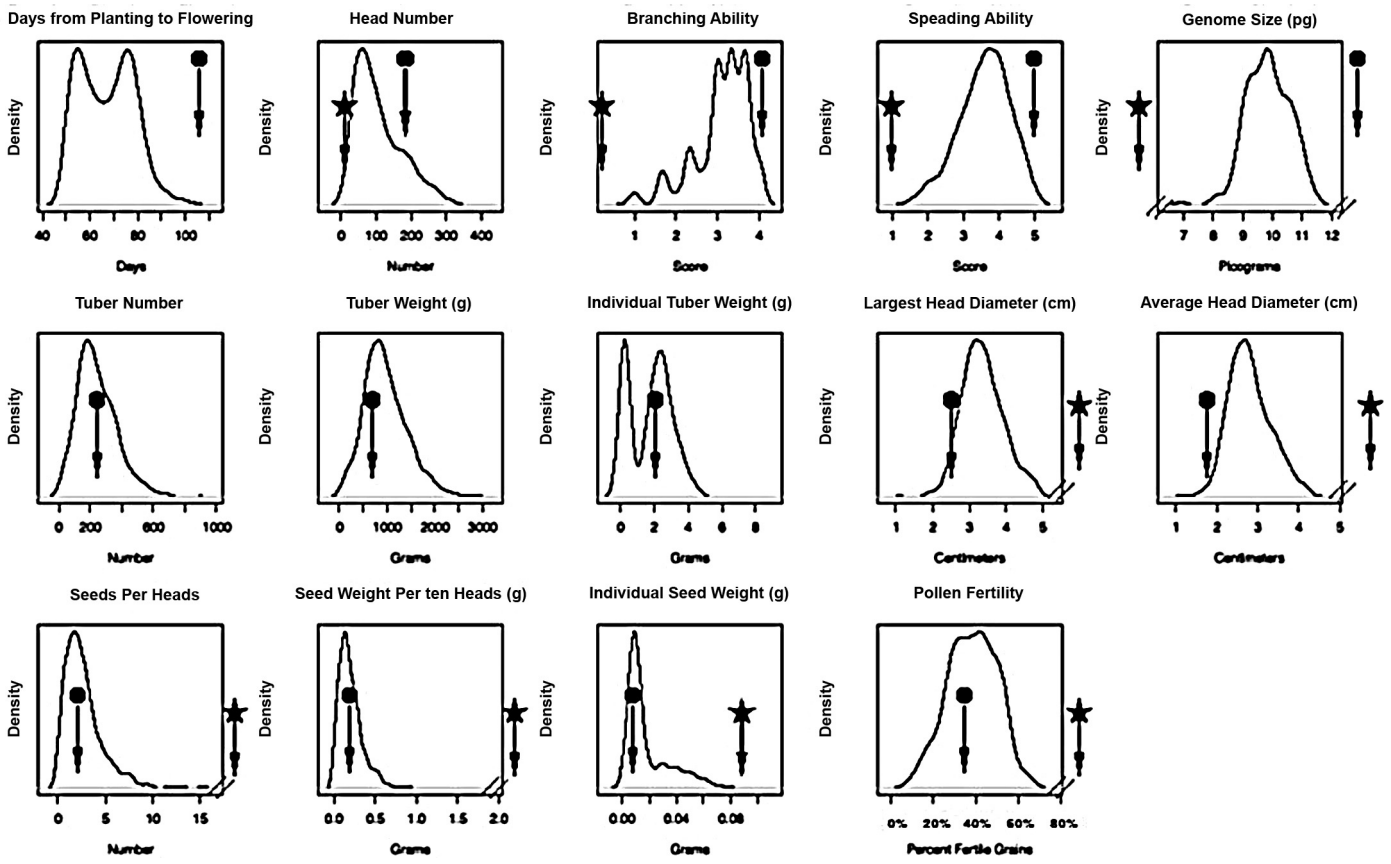

Fig. 2. Trait distributions in the F_1 hybrids. The symbol indicates where domestic annual *Helianthus annuus* is on the distribution.[1] The symbol ● indicates the trait value for wild perennial *Helianthus tuberosus* on the distribution with boxes under arrows indicating the value if it is outside the depicted distribution.

3.2. Trait evaluation in interspecific H. annuus x H. tuberosus F_1 populations

Generally, F_1 individuals were intermediate to the domesticated annual and the wild parents for each trait examined, with most traits having wide distributions but being more similar to the wild parent (Fig. 2; Supplementary Fig. 1). Some traits in the F_1 hybrids were more similar to the domesticated *H. annuus* (e.g., seed traits), and others were more similar to wild *H. tuberosus* (Fig. 2 and Table 3; Supplementary Fig. 1). For most traits, a few F_1 individuals closely resembled the domesticated phenotype. Across environments yield traits were positively correlated and tuber traits were correlated with each other (Supplementary Table 2). Seed weight and number were not correlated with tuber traits (Supplementary Table 2). Correlations from individual environments were different from the correlation pattern across all environments (Supplementary Table 2). For individual traits, heritability in the F_1 varied from 0.05 to 0.76 indicating differing selective potential for different traits in this breeding program (Supplementary Table 3). For most traits, heritability was similar across environments, but varied for seed weight and tuber weight (Supplementary Table 3). We observed individuals that were consistently superior for seed and agronomic traits compared to the wild *H. tuberosus* parent in all environments, particularly we observed increases in head size and seed yield.

F_1 plants typically flowered earlier than *H. tuberosus* (Fig. 2; Supplementary Table 4). Three architecture traits were measured: branching type, spreading ability, and head number. The F_1 hybrids were not statistically different from *H. tuberosus* for spread ability but were for branching type. While branching type was significantly different (less branching) between F_1 and *H. tuberosus*, it still was not unbranched like the domestic *H. annuus* (Fig. 2). F_1 plant spreading and head number varied greatly between environments. Hybrids displayed fewer total flowers, but not the single head type preferred in *H. annuus* cultivars. Three tuber traits were examined: tuber number, total tuber weight and individual tuber weight. The F_1 hybrids had greater tuber number, individual and total tuber weight than *H. tuberosus* (Table 3).

The F_1 families were different from their wild *H. tuberosus* parents for most yield traits (Table 3 and Fig. 2; Supplementary Table 4). Head size exhibited a wide phenotypic range both within plants and among families. The largest heads among the F_1 individuals were 5-6 times bigger in diameter than *H. tuberosus* (Fig. 2; Supplementary Fig. 1). The majority of F_1 plants had few seeds (0-25), yet some individual F_1 plants yielded ten times this amount. There was differential shattering or predation among environments that likely increased seed yield variability.

3.3. Trait evaluation in the IM_1F_1 population

F_1 individuals were intermated to generate an IM_1F_1 population. We continued to observe improvements for yield traits in this generation, despite the absence of artificial selection pressure in choosing parents for the IM_1F_1. However, differences in pollen fertility may indicate that inadvertent selection for viability of pollen occurred. In 2010, 55 IM_1F_1 plants were evaluated for head and

[1] Pollen fertility, genome size, largest head diameter, and average head diameter were measured in rows of *H. annuus* planted adjacent to this experiment, while seed per head and seed weight per ten head were known to be outside the depicted distribution based on previous published and unpublished experiments.

Table 3
Analysis of Variance for the phenotypic traits of the interspecific F_1 hybrids and *H. tuberosus* parents.

	Yield traits										Tuber traits					
	Largest head diameter		Average head diameter		Seed per head		Seed weight (g)		Individual seed weight (g)		Pollen fertility		Tuber number		Tuber weight (g)		Individual tuber weight (g)	
	DF	Sig.	DF	Sig.	DF	Sig.	DF	Sig.	DF	Sig.	DF	Sig.	DF	Sig.	DF	Sig.	DF	Sig.
Environment	2	**	2	**	2	ns	2	ns	2	ns	2	ns	2	**	2	**	2	**
Replication (environment)	6	**	6	*	6	**	6	ns	6	*	6	**	6	**	6	**	6	**
Genotype	183	**	183	**	183	**	183	**	183	**	183	**	185	**	185	**	185	**
H. tuberosus	17	ns	17	ns	17	ns	17	ns	17	ns	17	ns	17	ns	17	ns	17	ns
F_1	165	**	165	**	165	**	165	*	165	**	165	**	165	**	165	**	165	**
H. tuberosus half-sib family	16	ns	16	*	16	ns	16	ns	16	ns	16	*	16	ns	16	ns	16	ns
H. annuus half-sib family	2	**	2	**	2	**	2	**	2	ns	2	**	2	ns	2	ns	2	**
Full-sib family	23	**	23	**	23	**	23	ns	23	ns	23	**	23	**	23	**	23	ns
H. tuberosus vs. F_1	1	**	1	**	1	ns	1	*	1	ns	1	**	1	**	1	**	1	**
Genotype × environment	364	ns	364	ns	365	ns	365	ns	365	**	366	**	366	ns	366	ns	366	ns
Environment × *H. tuberosus*	34	ns	34	ns	34	ns	34	ns	34	ns	34	**	34	ns	34	ns	34	ns
Environment × F_1	328	**	328	**	329	**	329	ns	329	ns	330	**	330	**	330	**	330	ns
Environment × *H. annuus* half-sib family	4	*	4	ns	4	ns	4	*	4	ns	4	ns	4	*	4	ns	4	*
Environment × *H. tuberosus* half-sib family	32	ns	32	*	32	ns	32	**	32	ns	32	*	32	*	32	**	32	ns
Environment × Full-sib family	46	**	46	*	46	**	46	**	46	ns	46	ns	46	**	46	ns	46	**
Environment × (*H. tuberosus* vs. F_1)	2	**	2	**	2	**	2	ns	2	ns	2	**	2	ns	2	ns	2	ns
Error	761		762		915		915		915		1006		1035		1035		1033	

ns, not significant.
* Significant a $p \leq 0.05$.
** Significant a $p \leq 0.05$.

seed traits. Three plants were ranked in the top 10% for largest head diameter, seed weight, and seed per ten head for the IM_1F_1 population. The largest head was 20% larger than any head observed in the F_1. Positive correlations between head size, seed weight, and seed per head were maintained. The best IM_1F_1 individuals exhibited numerically higher values than the best F_1 individuals for all seed traits, indicating the potential power of a recurrent selection program.

While all F_1 individuals exhibited perenniality through tuber sprouting, the IM_1F_1 population segregated for the perennial habit. In the winter of 2009-2010, 71 plants were screened for winter survival with 77% (55) surviving, which did not differ from a 3:1 ratio ($p = 0.89$) but did differ from other tested ratios (Table 4). Initially, it was unknown whether this was a result of segregation for tuber production, tuber survival (winter hardiness), or both. To confirm that the 3:1 segregation result was largely the result of tuber production segregation, 151 additional IM_1F_1 plants were grown in the greenhouse during the winter of 2010-2011 and screened for tuber production. All control *H. tuberosus* plants and 67% (104) of the IM_1F_1 produced tubers. Again, the IM_1F_1 segregation did not significantly differ from a 3:1 ($p = 0.39$) ratio (Table 4). In 2011-2012, we grew these 104 tuber-bearing plants in the field at Rosemount and found that 51% died, largely attributable to winter kill. Detailed evaluation of tuber winter hardiness has not been conducted to identify the factors involved.

3.4. Parental diversity

Sixteen SSR markers were used to examine the genetic diversity in the parents of the Minnesota perennial sunflower breeding program and a subset of the accessions in the GRIN collection. Although diversity measures showed a moderate level of diversity (Supplementary Table 5), they are difficult to interpret due to complexities related to polyploidy (Brown and Young, 2000; Luo et al., 2006; Akhunov et al., 2010; Stift et al., 2008). However, useful estimates of the relatedness of the breeding material to other germplasm can be made. The parents of the Minnesota breeding program did not cluster with the accessions from the GRIN collection (Fig. 3). R_{ST} indicated that the Minnesota population is moderately different than the GRIN accessions when grouped as a population (Supplementary Table 6). Structure analysis identified two subpopulations ($K = 2$) as the best fit, essentially dividing the Minnesota and GRIN accessions (Fig. 3a) (Pritchard et al., 2000; Evanno et al., 2005). This was similar to the interpretation obtained from examining a phylogenetic tree based on genetic distance (Fig. 3b). This indicates that the 18 *H. tuberosus* individuals used to develop the F_1 and IM_1F_1 populations represent a relatively narrow sampling of the genetic pool of the species.

4. Discussion

4.1. Prospects and limitations for perennial grain breeding

The combination of traits comprising the perennial *Helianthus* seed crop ideotype includes seed and head traits contributing to high seed yield (high pollen fertility, high seed weight, large head size), plant architecture traits enabling uniform seed maturity (no branching and one central flower, or all heads having synchronous flowering), and tuber traits contributing to a manageable perennial habit (low tuber number, high individual tuber weight). Therefore, the perennial ideotype is the domesticated phenotype with the addition of perenniality. This ideotype for the initial perennial sunflower lines would be targeted toward marginal landscapes with high potential for degradation to maximize the environmental benefit. In addition, the lines could be used as a trap crop near production fields to help mitigate bird predation.

Table 4
Segregation ratios for tuber production in IM_1F_1 plants.

	3:1 ratio for tuber production		9:7 ratio for tuber production	
	Winter 2009–2010	Winter 2010–2011	Winter 2009–2010	Winter 2010–2011
Plants examined	71	151	71	151
Observed number of tuber producers	55	104	55	104
Expected number of tuber producers	53.25	113.25	39.94	84.94
Chi-square value	0.019	0.75	5.63	4.24
p-Value	0.89	0.39	0.02	0.04

The straightforward way to produce a perennial sunflower would be to backcross perennial habit into annual sunflower. Based on our investigation, we found that when F_1 plants (tetraploid) were crossed with *H. annuus* plants (diploid), weak annual triploid plants were generated. This was also found in other species where backcrossing approaches have generally led to a loss of perenniality, so breeding programs have focused on domestication of interspecific hybrids or wild relatives (Cox et al., 2010). Therefore, we refocused our efforts on selection and intermating of the best F_1 individuals.

From a physiological perspective, there is reason for optimism regarding our refocused efforts on selecting for domestication phenotypes in the intermated individuals. With the potential to allocate resources to both sexual and asexual reproduction, the expectation is that both types of reproduction will compete for resources (Darwin, 1876; Van Noordwijk and De Jong, 1986). Empirical studies have identified negative (Westley, 1993) and positive phenotypic correlations (Cheplick, 1995) between sexual and asexual reproduction, with occasional genetic correlations as well as large environmental effects on resource allocation (Westley, 1993; Piquot et al., 1998). However, we observed few negative correlations between tuber and seed traits with total seed weight being significantly positively correlated with tuber traits; this may be due to the resource rich environments in which the plans were grown (Cheplick, 1995). The wide phenotypic distributions (with some favorable types and transgressive segregants) in the F_1 hybrids described here indicate that selection for both perenniality and yield in this population may be possible. Furthermore, wider ranges were observed in the IM_1F_1 compared to the F_1 for some traits, indicating that recurrent selection on intermated materials may successfully take advantage of the wide genetic variation for further improvement. Key improvement targets for the breeding program are summarized in Table 5.

From a genetic perspective, the situation is more nuanced and depends on currently unknown factors. It is attractive to consider the past utilization of the *Helianthus* wild germplasm as an indicator of future success for introgressing perenniality. Additionally, Baack et al. (2008) made the promising observation that selection for domestication traits proceeds rapidly in progeny from domestic × wild annual *Helianthus* matings. We have observed this first hand, as improvements in seed and head traits were achieved while maintaining perennial habit after one generation of intermating. However, our breeding design is unique in that it uses interploidy hybridization and maintains the populations as polyploids. The ability to drive the phenotypic traits toward the ideotype using our intermating scheme will depend on the rate at which the *H. tuberosus* genetic material can be purged from the genome (and replaced by the *H. annuus* genetic material), while maintaining perenniality. There are two factors that will determine this limitation: (1) the amount of *H. tuberosus* genetic material that is required for perenniality and (2) the meiotic pairing behavior of homeologous chromosomes.

Best case scenarios require that very little *H. tuberosus* genetic material is necessary to confer perenniality in the intermated progeny. This would reduce the linkage drag associated with introgressing the perenniality loci. Furthermore, an ideal scenario would presume that the A_1, A_2, and B_t sub-genomes of *H. tuberosus* are all capable of pairing with the *H. annuus* chromosomes. In this case, it would be possible to continuously increase the proportion of the *H. annuus* genome with each successive generation of intermating possibly increasing the speed of producing the ideotype. However, if the B_t genome exclusively pairs with the *H. annuus* chromosomes, then it would be impossible to purge the A_1 and A_2 chromosomes, regardless of the number of generations of intermating. In this case, all of the intermated tetraploid plants would contain at least 50% *H. tuberosus* genome, severely limiting the progress that could be made toward achieving the ideotype.

If conventional breeding is severely limited by these genetic limitations, it may be possible that genetic transformation could provide an avenue to create a plant more similar to the ideotype. If the gene(s) underlying the seemingly simple segregation in the IM_1F_1 for tuber development (discussed in the next section) can be identified, they may be cloned and transformed for this purpose. It is unclear if such an approach would be acceptable to consumers, and the approach is likely to be subject to regulatory assessment due to the invasive potential of transgenic sunflowers. The intriguing question also arises whether coupling genetic transformation to ecosystem services will make the technology more palatable.

4.2. Segregation of perenniality in Helianthus IM_1F_1 and other species

Variation in perennial traits (including the lack of perenniality) has been reported in populations derived from perennial rice, sorghum, wheat grass, and teosinte crossed with their annual crop relatives (Hu et al., 2003; Murphy et al., 2009; Lammer et al., 2004; Westerbergh and Doebley, 2004; Sacks et al., 2003a,b, 2006a,b). In the present study, all *Helianthus* F_1 plants were perennial and the IM_1F_1 population segregated approximately 3:1 for tuber production with tuber survival (over-wintering ability) differing among seasons. This finding implies that it may be relatively simple to identify the genetic factors that are most essential for perennial organ development.

Segregation for perenniality in the IM_1F_1 has several potential explanations (Fig. 4). The first and simplest explanation is that a single dominant gene in the *H. tuberosus* genome is necessary for tuberization (Fig. 4b), but it is probably not that simple because the BC_1F_1 generations did not produce tubers. Second, segregation for perenniality may result from sub-genome dosage effects. It has been observed that interspecific hybrids with genomic composition less than 50% of the perennial parent rarely maintain pereniality (Cox et al., 2002, 2010). This suggests that there may be a stoichiometric regulatory balance, and that certain proportions are necessary for phenotypic stability (Birchler and Veitia, 2001; Birchler et al., 2001). This scenario is supported by the lack of perenniality in the BC_1F_1 individuals, which had a reduced relative proportion of the *H. tuberosus* sub-genomes (Fig. 4c). This may present a problem as we continue to attempt to enrich for rare recombination events to purge as much as possible of

Table 5
Moving the University of Minnesota perennial sunflower breeding program toward the perennial ideotype.

Trait	Ideotype	Elite F_1	IM_1F_1	Potential
Flowering time	• Early to intermediate	• Resembled the domestic type in flowering time	• Flowering was similar to F_1	• Flowering time was identified as an important domestication trait with initial domestication favoring early flowering (Blackman et al., 2011) and is already starting to resemble the ideotype
	• Flowering time during the year			
Plan architecture	• No branching	• Some branching	• Less branching than F_1	• Extreme individuals and moderate heritability for architecture traits indicate that selection for improved types is possible
	• Single head	• Multiple heads	• Fewer flowers than F_1	• Flower number may be under relatively simple genetic control (Hockett and Knowles, 1970; Putt, 1964)
	• Minimal Spread	• Exhibited heterosis for biomass leading to more spread and vigor relative to the H. tuberosus plants	• Variation similar to the F_1 but had more extreme types for plant spread	• Spreading of interspecific hybrids showed variation, including types that did not spread indicating progress toward the ideotype
Tuber traits	• Few tubers	• High tuber numbers	• Variation similar to the F_1	• Tuber number had high narrow sense heritability, indicating that genetic effects may be easy to select for and that it may be easy to select individuals with low tuber number
	• Intermediate tuber yield high	• Heterosis for tuber yield		• Not all F_1 may be persistent and genotypes can be selected for decreased weed potential as individuals with a phenotype similar to the perennial ideotype were identified
	• Individual tuber weight • This would be ideal because larger tubers would reliably germinate but if numbers were low there would likely be minimal spread	• Tubers were generally small		
Yield traits	• Large headed like *H. annuus*	• Low family means compared to the range for yield traits with many individual outliers, similar to interspecific perennial rice populations (Sacks et al., 2006a)	• Variation similar to the F_1 but had more extreme individuals	• There was no relationship between pollen fertility and perennial traits, mirroring studies in interspecific rice (Sacks et al., 2006a)
	• Large number of seed per head			• Pollen fertility was not a goo predictor of yield, likely as here is little cost to the plant, as increased pollen production can account for low viability
	• High total yield			• In the F_1 tuber and yield traits were not correlated indicating that there may not be an antagonistic relationship between perenniality and yield
	• High individual seed weight			• Head size had a high narrow sense heritability indication selection for larger heads may progress rapidly
	• High pollen fertility			• The low narrow sense heritability in seed weight indicates that many genes and/or genes with large non-additive effects are involved in its control (Fehr, 1991).
				• Progress has been made toward he perennial ideotype

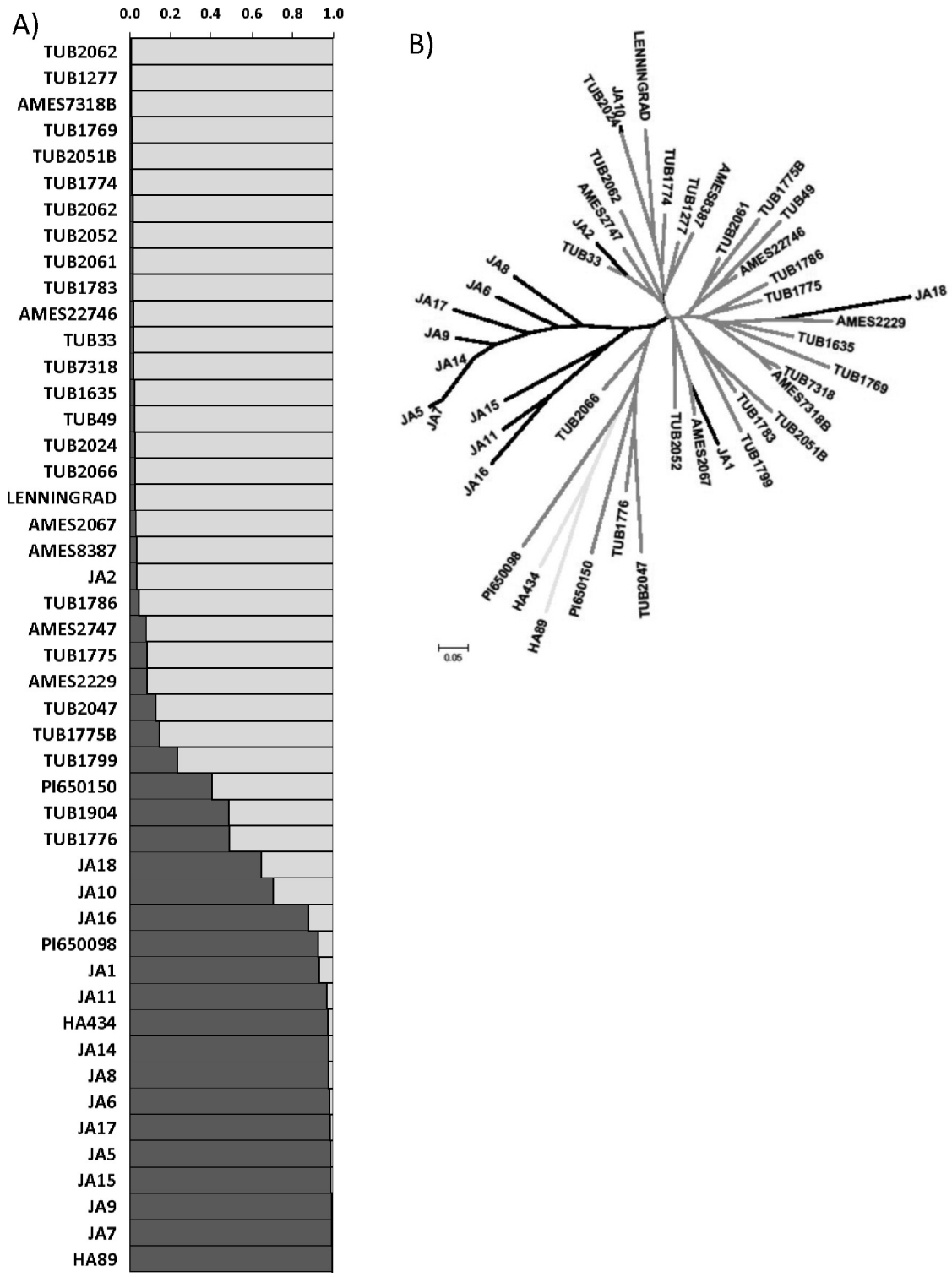

Fig. 3. (A) Structure clustering based on the same 16 SSR markers with colors indicating different sub-populations (light gray indicating membership in the GRIN collection and dark gray indicating membership in the Minnesota wild collected material), partial coloring indicated mixed ancestry for an individual accession, with percent membership indicated by the axis. (B) Neighbor Joining Tree based on 16 SSR markers. Black indicates *H. tuberosus* parents within the University of Minnesota perennial sunflower breeding program, light gray indicates *H. annuus* parents within the University of Minnesota perennial sunflower breeding program, and gray indicates accessions from the GRIN database.

the *H. tuberosus* genome, but currently we see improvement in phenotypes. Thirdly, the interspecific hybrid may be viewed as a neopolyploid, which may cause multivalent pairing, homoeologous recombination, aneuploidy, and/or large de novo structural variants (Chester et al., 2012; Tate et al., 2009). If this scenario is true genome stability may be increased by intermating for several generations. While these phenomena may result in inconsistent transmission for many traits, including perenniality, imposing selection may lead to more stable plants allowing us to identify those individuals that have the perennial ideotype.

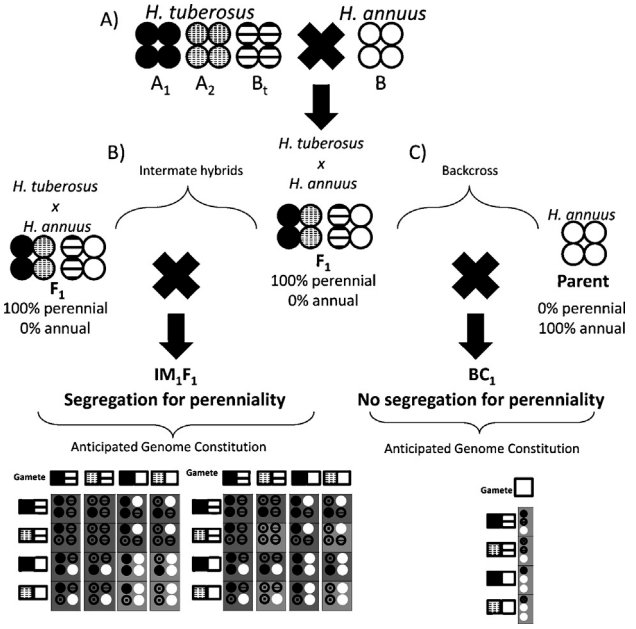

Fig. 4. Hypothetical models of chromosome segregation that lead to perennial and annual progeny. (A) *H. annuus* × *H. tuberosus* hybridization leads to 100% perenniality. White indicates the *H. annuus* chromosomes; chromosomes from the three sub-genomes of *H. tuberosus* are indicated by solid black (A_1), checkered (A_2) or lined (B_t) patterns. (B) Subsequent intermating of F_1 hybrids yields progeny that segregate for perenniality. Dark gray background indicates the genotype for perennial plants and light gray indicates the genotype for annual plants. Two models are shown to explain the ~3:1 segregation pattern. The model on the left associates perennialty with the dosage of the *H. tuberosus* chromosomes relative to the *H. annuus* chromosomes (annual plants exhibit a higher dosage of *H. annuus* chromosomes). The model on the right associates perennialty with a single factor sufficient for tuber production segregating from one of the *H. tuberosus* sub-genomes. In this example, the single factor resides on one of the A_1 chromosomes. (C) Backcrossing the F_1 hybrid with *H. annuus* yields progeny that are all annual. This result is consistent with both the dosage model or the single segregating factor model described in part (B).

5. Conclusion

Based on the examination of the *H. tuberosus* parents, the F_1, IM_1F_1 and BC_1F_1, hybridization followed by selection for domestication traits appears feasible to improve *Helianthus* for use as a perennial oil-seed crop. The development of perennial oil-seed is a long term endeavor; however, there are checkpoints along the way such as use as a trap crop that provide value during the development. As these checkpoints are reached new agronomic and disease challenges (likely due to the lack of crop rotation) will need to be addressed in order for perennial crops to be adopted. In addition, much can be learned about the biology of perennial habit and about interspecific hybridization. The intermating (IM_1F_1) approach exhibited the greatest potential, as domestication traits were improved in the IM_1F_1 while maintaining perenniality in a high proportion of the population. The improved phenotypic traits in the IM_1F_1 may be indicative of the loss of wild chromosomes or portions of chromosomes in favor of domestic chromosomes. Therefore, recurrent intermating and selection of advanced intermated lines appears to be a promising approach for further improvement. Perenniality may segregate in a relatively simple way even if the underlying genetics are complex. Our data indicate that we have started our program with limited diversity, but despite this we have seen gains in initial generations (Table 5; Supplementary Table 4). If we do not see continued gains, the addition of more parents to the University of Minnesota perennial sunflower breeding program would likely be beneficial. There is potential to eventually develop a perennial *H. annuus*-like plant that produces tubers and yield grain consistently over the life of a stand, leading to a crop that produces ecosystem services while having a commercially viable yield.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2013.04.018.

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